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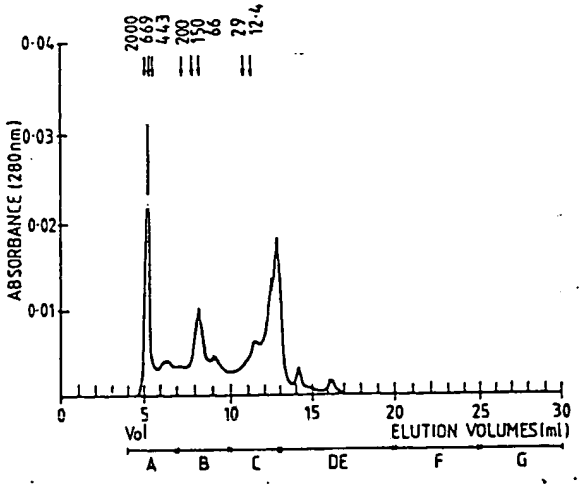

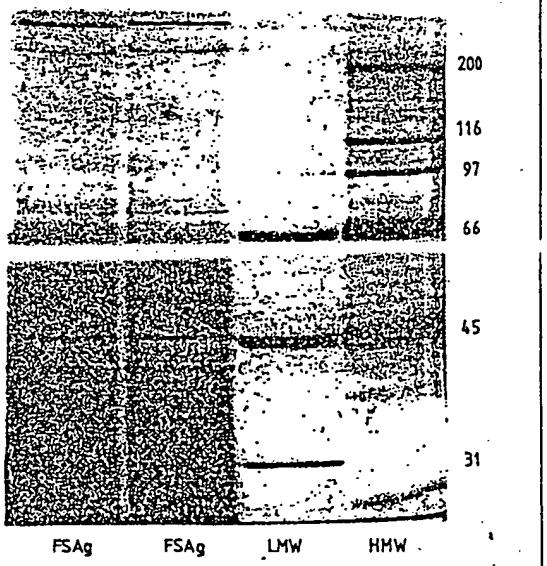

(57) Abstract

The invention provides an antigen preparation from fleas of the genus *Ctenocephalides* characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 anda denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5. The invention also provides a method of preparing the antigen preparation. The antigen preparation finds use in a method for testing for flea allergy in animals, particularly for the diagnosis of flea allergy dermatitis in dogs and cats. The antigen preparation may also be used for hyposensitising allergic animals.

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TITLE

"AN ANTIGEN FOR DIAGNOSIS AND HYPOSENSITISATION OF FLEA ALLERGY DERMATITIS IN ANIMALS".

FIELD OF THE INVENTION

THIS INVENTION relates to an antigen and method for detecting allergic dermatitis in animals caused by reactivity to fleas. In particular, the invention relates to determining whether dermatitis in pets such as dogs and cats is caused by fleas, by using a soluble flea antigen in intradermal skin tests to establish the presence or absence of a reactivity to this antigen. Reactivity to this flea antigen is indicative of its potential for hyposensitisation (i.e. therapy) of the animal.

BACKGROUND OF THE INVENTION

It is well known that the bite of the flea causes a hypersensitivity in some animals which manifests as a disease termed, most usually, flea allergy dermatitis (FAD). FAD is the most common cause for presentation of dogs to veterinarians in the USA (Halliwell 1986) and is found worldwide in cats and dogs exposed to fleas; this disease causes major irritation (Baker and Hatch 1972, Kieffer and Kristensen 1979). Naive and non-sensitised animals have little reaction to fleabites, but those animals which are hypersensitive develop an erythematous wheal (type I or immediate type hypersensitivity) which may resolve and be followed by development of a papule with a crust (delayed type hypersensitivity). The lesions are nighly pruritic and the animal frequently traumatizes itself with subsequent dermatitis, erythema, hair loss, acanthosis, lichenification, and pyoderma developing. The clinical signs of canine and feline FAD have been well described in the literature (Doering 1976; Ihrke 1983; Shaw 1987; Dryden and Blakemore 1989; amongst others).

Fleas are the most prevalent insect parasite of pets worldwide (Reedy 1986) and FAD is, in the main, caused by the cat flea *Ctenocephalides felis* (Dryden and Blakemore 1989). The next most prevalent flea is *Ctenocephalides*

canis although it's prevalence is significantly lower than *Ctenocephalides felis*.

The immunopathology of FAD is poorly understood. The standard sequence of reactions which occurs in mammals in response to bites by arthropods is not found in FAD in dogs or cats. Flea naive dogs experimentally exposed to fleas developed a type I hypersensitivity at 15 minutes with eosinophilic and oedematous infiltrates in the perivascular region of the skin. A delayed reactivity occurred at 24 hours characterised by a mononuclear cell infiltrate and the continued presence of eosinophilic leukocytes (Gross and Halliwell 1985). The delayed type hypersensitivity (DTH) was proposed to be a cutaneous basophil hypersensitivity (CBH). Later work by Halliwell showed that biopsies taken from the sensitisation site contained large numbers of basophilic leukocytes in flea allergic dogs (Halliwell and Schemmer 1987). It is likely that the delayed response is a mixture of classic DTH and CBH.

Halliwell and Longino (1985) demonstrated the presence of IgE antibodies specific for flea antigens in the sera of dogs with FAD. These dogs also had high levels of IgG anti-flea antibodies. Non-allergic dogs which had been exposed to fleas had low levels of both types of antibodies; it was suggested that dogs continuously exposed to fleas were tolerized and unable to react to fleas.

It is unclear why some animals develop FAD whereas others although exposed to fleas do not. Factors which affect the propensity of a dog or cat to develop FAD include intermittent exposure to fleas (Halliwell 1984); type of housing, e.g. maintained indoors; age of first exposure to fleas (Halliwell et al 1987); and the presence of atopy.

A diagnosis of FAD is suggested by dermatitis associated with the presence of fleas, flea excreta or flea eggs on the pet, and/or finding *Dipylidium caninum* segments in the faeces or anal region. FAD is also diagnosed by using an intradermal skin test (IDST) where

flea antigens are injected intradermally or intracutaneously and the responses of the animal (type I and/or type IV hypersensitivity) are monitored at intervals ≤ 1 hour and at 24 - 72 hours respectively. A standardized test for FAD does not exist and there is controversy on the value of the flea extracts available for use in these tests.

For example, Baker (1971) evaluated the responses of dogs with and without clinical signs of FAD to injection with an aqueous extract of fleas. He graded their responses subjectively 15 minutes later and found 74% of dogs with FAD reacted to the antigen and 6% of dogs without FAD responded. However other authors (Kieffer and Kristensen 1979) using a similar flea extract and an objective method (measurement of wheals) for evaluating responses, found that 15% of control dogs were false positive to the test. They were able to detect a similar number (76%) of true positive results. The antigen did not detect cats with FAD very well (35%).

Glycerinated antigens were concluded by several authors to give unacceptably high false positive results (Nesbitt and Schmitz 1978; Van Winkle 1981).

In 1981 Van Winkle tested 4 flea antigen preparations which are commercially available for diagnosing FAD. These included: the antigen manufactured by Center Laboratories (Port Washington, New York); Haver-Lockhart antigen (Kansas City, Missouri); Hollister-Stier antigen (Spokane, Washington); and Greer antigen (North Carolina). He found all 4 antigens unreliable although Greer antigen proved the best. In contrast Halliwell (1981) and MacDonald (1983) recommended the use of Greer antigen for diagnostic purposes with MacDonald (1983) claiming that 90% of FAD cases could be detected using this antigen in IDST. In a later study Reedy (1986) showed that of 193 cases suspected of having FAD only 45% could be identified using Greer antigen. Another commercial flea antigen available is that produced by Nelco Labs (New York) and Lorenz (1980) showed that only one quarter of clinical

FAD cases could be identified with this antigen. Indeed, later Lorenz (1984) stated that this antigen was "not superior to the diluent control in predicting reactivity".

It is clear from the information available in the literature that the flea antigen preparations available for the diagnosis of FAD have a variable efficacy and indeed Reedy in 1986 reporting on IDST studies with Greer antigen suggested that the percentage positive results "will differ considerably with exposure and in different parts of the country". Clearly there is a need for a reliable antigen for use in FAD diagnosis and in hyposensitisation.

The results of hyposensitisation trials to date have been equivocal. Several studies have been carried out (Keep and Taylor 1967; Nesbitt and Schmitz 1978; Halliwell 1981; and Kunkle and Milcarsky 1985). The earlier studies either did not use control groups or carried out rigorous flea control at the same time as hyposensitisation therapy thereby obscuring the results of the hyposensitisation therapy. The results from the later studies where double blind conditions were applied, did not support hyposensitisation as a treatment for FAD.

From a consideration of antigen preparations known to be useful in IDST techniques, those of skill in the art would predict that membrane containing preparations would be the most suitable for a flea allergy test in animals. The present inventors have surprisingly found that a soluble, membrane free, preparation provides superior results in such a flea allergy test.

OBJECTS OF THE INVENTION

It is therefore an object of the invention to provide an antigen for diagnosis of FAD in animals such as dogs and cats.

It is a further object of the present invention to provide a method of diagnosis of flea allergy in domestic animals such as dogs and cats.

SUMMARY OF THE INVENTION

According to one aspect of the present invention

there is provided an antigen preparation from fleas of the genus *Ctenocephalides* characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5.

Preferably the antigen preparation is further characterised as having an IgG immunoblotting pattern as shown in Figure 6 or an IgE immunoblotting pattern as shown in Figure 7.

Typically, the antigen preparation is from fleas of the species *Ctenocephalides felis* and *Ctenocephalides canis*

According to another aspect of the present invention there is provided an antigen preparation prepared by a process comprising the following steps:

a) preparing a total extract of fleas of the genus *Ctenocephalides*;

b) removing debris and cells from said total extract to provide a whole flea extract; and

c) separating a soluble flea antigen preparation from said whole flea extract.

A still further aspect of the present invention comprises a method of detecting flea allergy in an animal, said method comprising the steps of:

a) intradermally injecting into an animal an antigen preparation from fleas of the genus *Ctenocephalides* characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5;

b) measuring the diameter of the wheal formed at the site of injection; and

c) comparing said wheal diameter with the diameter of wheals formed at the site of injection of a control solution to determine whether said animal is allergic to said fleas.

A still further aspect of the present invention comprises a method of hyposensitising an animal to flea allergy comprising administering to said animal an

antigen preparation from fleas of the genus *Ctenocephalides*, said antigen characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5.

The antigen preparation is particularly effective against the species *Ctenocephalides felis* and *Ctenocephalides canis*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 - Profile on high performance size exclusion chromatography (HPSEC) of soluble flea antigen (FS).

Figure 2 - Diameters of wheals obtained on titration of FS antigen in dog groups which reacted positively (—■—) and negatively (—▲—) to flea-feeding and for pups (—□—). Wheals were measured 15 minutes after intradermal injection of the antigen. The group mean of the average wheal diameter minus the average wheal diameter of the negative (buffer) control was established for each antigen concentration. Bars represent the SEM.

Figure 3 - IgG antibody levels against WF, FM, FS and c-antigen in the sera of three groups of dogs: dogs reacting positively to flea-feeding (), dogs which did not react to flea-feeding () and pups (). Antibodies were measured in an ELISA. The mean ± SEM (represented by bars) absorbance values at 405 nm are shown for each group of dogs.

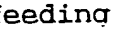
Figure 4 - IgE antibody levels against WF, FM, FS and c-antigen in the sera of three groups of dogs: dogs reacting positively to flea-feeding (), dogs which did not react to flea-feeding () and pups (). Antibodies were measured in an ELISA. The mean ± SEM (represented by bars) absorbance values at 405 nm are shown for each group of dogs.

Figure 5 - Stained denaturing polyacrylamide gel of electrophoresed antigen preparation. FS-Ag, antigen preparation; LMW, low molecular weight standards; HMW, high molecular weight standards.

Figure 6 - IgG immunoblot result for six FF+ and six

FF- dog sera. Lanes labelled 1-6 contain dog sera from FF+ dogs arranged in order of decreasing (IgG) vs FS-Ag: lanes 7-12 contain sera from FF- dogs arranged in a similar manner.

Figure 7 - IgE immunoblot result for six FF+ and six FF- dog sera. Lanes labelled 1-6 contain dog sera from FF+ dogs; lanes 7-12 contain sera from FF- dogs.

DESCRIPTION OF PREFERRED EMBODIMENTS

Preferred aspects of the present invention are described in the following preferred embodiment which relates to an appropriate experimental procedure.

Frozen adult fleas, fed or unfed, preferably but not exclusively *Ctenocephalides felis*, about 5,000 to 7,000 are homogenized in preferably 0.15m phosphate buffered saline, pH 7.2 (antigen buffer) or other suitable buffer by shaking vigorously in a disintegrator containing 4 mm diameter stainless steel beads. Any other suitable method of external pressure may also be employed to disrupt fleas. Disrupted fleas are further disrupted by exposure to Ultrasound using a sonicator for periods typically up to 5 minutes. The temperature of the homogenate is maintained as close as possible to 4°C throughout and is not allowed to exceed 12°C. Protease inhibitors, e.g. Disodium EDTA, phenylmethylfluorane sulphonate amongst others, may be incorporated into the antigen buffer during disruption phases. The mixture is centrifuged to pellet unbroken cells and organelles, typically at 600 g for 10 minutes, and the pellet may either be resonicated or discarded. The supernatant from the first sonication procedure and the resuspended sonicated pellets are pooled and centrifuged, typically at 15,000 g for 20 minutes, to remove unbroken cells debris and organelles. The supernatant is then centrifuged at 100,000 g for 1 hour and the supernatant following this step is designated soluble flea antigen (FS). FS antigen can be characterised and controlled for consistency of content between extracts by separating the

preparation by high performance size exclusion chromatography (HPSEC). Chromatography is carried out typically, but not exclusively, on a Waters Protein Pak SW300, 7.5 mm (ID) x 30 cm containing Protein I-125 and protected by a guard column. Chromatographic conditions are flow rate, 1 ml/min; injection volume 100 µl; chart speed 0.5 cm/min; run time 30 min; AUFS 0.1. Molecular weight markers from 12.4 to 2000 kilodaltons are used to establish the molecular weights of peaks. Three major and several minor peaks are obtained, these are shown in the figure below. All proteins are ≤ 2000 kDa. One of the protein peaks elutes below 12.4 kDa.

Protein concentrations of antigen preparations may be determined by any standard system used to assay protein but typically these concentrations are obtained using a modified Lowry assay (Markwell et al 1978).

This soluble flea antigen, FS or derivatives thereof are used in the IDST to establish the presence or absence of reactivity to flea antigens.

Typically the IDST is carried out as follows. Dogs may be anaesthetized for this procedure using an intravenous injection of Thiopentone sodium and cats may be sedated by intramuscular injection of Xylazine. The lateral chest may be clipped and injection sites marked with a felt tip pen. FS antigen, typically 60 µl but ranging from 20-100 µl and containing typically one µg protein per dose but a dose ranging from 0.05 µg to 5 µg protein per injection may be used. Antigen and control preparations are injected intracutaneously using a 27 gauge needle and a tuberculin syringe. A bleb at the site of injection indicates that the antigen has been deposited in the skin. Homologous buffer, filtered through a 0.45 µm membrane filter is used as a negative control. Histamine phosphate diluted 1/10,000 (w/v) or 1/100,000 w/v in filtered buffer is used as a positive control if desired.

The skin test readings may be assessed by a variety of objective grading systems. However, the preferred method of grading is taken as a positive reaction

occurring where the response to injection with antigen is 5 mm or more greater than the negative control preparation (buffer). The measurements of the wheal reaction are carried out 15 minutes after injection of the antigen and are typically made by measuring the horizontal and vertical diameter of the wheal and making an average. It is standard procedure to carry out tests in replicates (2 or 3 assays), particularly where the operator has limited experience with the assay system.

It will be appreciated that the scope of the present invention includes an antigen for diagnosis of FAD and also for hyposensitisation procedures using this antigen or derivatives thereof. FS may be utilised in regard to hyposensitisation using regimens which are standard for this procedure. These include but are not limited to intradermal injections at 2-10 day intervals with 10-1000 µl of antigen, administered at different sites on the body and continued until either a therapeutic effect becomes evident or blocking antibodies develop or specific T suppressor cells develop.

The present invention may be more fully appreciated by reference to the following set of examples labelled example 1 to example 4.

Example 1: FS used in IDST in a preliminary experiment in dogs.

MATERIALS AND METHODS

Animals - Dogs obtained from dog shelters in Brisbane, Australia, were examined for clinical signs of FAD and were evaluated for the presence of fleas and flea excreta as positive evidence of infestation. A group of 25 dogs was thus categorized as group 1: pups under 6 months, no evidence of skin lesions (n=6); group 2: over 6 months, evidence of dermatitis consistent with FAD (n=7); group 3: over 6 months, no evidence of FAD (n=11). The dogs were of mixed breeds, were aged from their dentition and were not matched for sex.

A flea colony was obtained (Bayer Australia Ltd, Beenleigh, Australia), identified as *Ctenocephalides felis felis* by morphological examination (Dr R. Boreham

pers. commun.) and ascertained to be free of *Dipylidium caninum*. The colony was maintained by passage of fleas on 3 cats. Flea eggs were collected and batches of about 500 eggs were incubated at 30°C for 14 to 28 days in the presence of 10g vermiculite, 2g brewer's yeast and 2g protein meal (Pugh, R. 1986 PhD thesis The University of Queensland, Australia). Adult fleas were harvested three times a week.

Antigens - Fleas were stored at -70°C and aliquots of frozen fleas were homogenized in 0.15M phosphate buffered saline (PBS), pH 7.2, by vigorous shaking for 4 minutes in a steel disintegrator using stainless steel beads (4 mm diameter); the disintegrator was cooled to 4°C using a water jacket. Homogenized fleas were sonicated (MSE Soniprep 150, CMS Inc., Houston TX, USA) at an amplitude of 21 microns in 30 seconds bursts for a total of 4 minutes, interrupted by periods for cooling. The mixture was centrifuged for 10 minutes at 600 g and the pellet resuspended in an aliquot of the supernatant and resonicated for 3 minutes as before. The total extract was then centrifuged for 20 minutes at 15,000 g to free the preparation from debris and cells and the resulting supernatant, designated whole flea extract (WF), was stored at -70°C. WF was also centrifuged at 100,000 g for 1 hour and separated into soluble flea antigens (FS) and flea membrane (FM). FM was resuspended in 10 mM phosphate buffer, pH 7.2 (membrane buffer). All procedures were done either on ice or at 4°C and antigens were stored at -70°C. Protein concentrations of antigen preparations were determined by a modified Lowry method. A commercial preparation of flea antigen (c-antigen) consisting of an aqueous whole flea extract (*Ctenocephalides spp*) preserved with phenol was purchased (Greer Laboratories Inc., Lenoir NC, USA).

Intradermal testing with flea antigens - Dogs were anaesthetized by i.v. injection with Thiopentone Sodium 2.5% (10-30 mg/kg bodyweight); the lateral chest was then clipped using #40 blades and injection sites marked off in 2 cm squares with a felt pen. Sixty µl of each

antigen dilution and control preparations were injected intradermally using disposable 1 ml tuberculin syringes and 27 gauge needles. Production of a bleb at the site of injection was taken to indicate that the antigen had been deposited in the skin.

C-antigen was tested in triplicate in each animal in a 1/1000 w/v dilution in accordance with the manufacturers directions. FM and FS were injected as 6 dilutions ranging from 1.25 µg to 0.01 µg/dose and 2 µg to 0.01 µg/dose, respectively. Sterile 0.15 mM PBS pH 7.2 was the diluent for all preparations and was also used as a negative control; 1/100,000 w/v histamine phosphate (DBL, Mulgrave, Victoria, Australia) was used as a positive control. All antigens and the positive control preparation were diluted just prior to use.

The vertical and horizontal diameters of wheals were measured at 15 minutes, 30 minutes, 1 hour and 24 hours after injection. The average wheal diameter of the negative control was subtracted from that of the injected antigen. A reaction was considered positive if the average wheal diameter exceeded that of the negative control by 5 mm (manufacturers directions for c-antigen). The order of administering injections of the various concentrations of FS was reversed in 3 dogs on the right versus the left side and the wheal diameters were compared; it was established thus that the site of injection did not significantly affect responses. The data obtained from intradermal tests were considered valid where the average wheal diameter of the positive control (histamine) exceeded the average wheal diameter of the negative control (buffer) by 5 mm; only dogs which conformed to these criteria were included for analysis.

Feeding of fleas on dogs (flea provocation test) - Fleas (15) harvested on the day of use were counted into clear, round plastic containers (diameter 27 mm, height 68 mm) which were then covered with taut muslin. Each dog was exposed to fleas by tight apposition of the muslin covered opening of the plastic container to the clipped lateral chest for 15 minutes. The number of fleas which

fed was counted by dissecting the guts from the fleas under a dissecting microscope and evaluating the guts for presence of bloodmeal. The site of feeding was examined 15 minutes, 30 minutes, 1 hour and 24 hours after exposure and recorded as positive where palpable and visible wheals occurred.

Serology - Blood samples were taken from the jugular vein of all dogs and serum was prepared and stored at -20°C. Anti-flea antibodies were measured by an indirect enzyme-linked immunosorbent assay (ELISA). WF (5 µg/ml), c-antigen (diluted at 1/18), FM (10 µg/ml) and FS (5 µg/ml) in 0.06M carbonate buffer, pH 9.6, were used to coat microtitre plates. Reactive sites on the plates were blocked with 1% gelatine in carbonate buffer. Sera were serially diluted in 0.15M PBS pH 7.2, containing 0.05% Tween 20. To assay IgG levels of anti-flea antibodies, goat anti-dog IgG (heavy and light chains specific) conjugated to peroxidase (Cappel, Cooper Biomedical, PA USA) was used as the marker antibody and 2,2-azino-bis-(3-ethylbenzthiazoline 6-sulfonic acid) diammonium (ABTS) as the substrate (Sigma Chemical Co., St Louis, MO USA). To assay the IgE levels of anti-flea antibodies, goat anti-dog IgE conjugated with peroxidase (supplied as a gift by Kirkegaard & Perry Laboratories Inc, Gaithersburg MD, USA) was used after blocking cross-reactivity to IgG using an excess of goat anti-dog IgG (heavy and light chains specific) (DBL, Mulgrave, Victoria, Australia).

Plates were read at 405 nm on an automatic ELISA plate reader (Titertek Multiskan, Flow Laboratories, Ayrshire, Scotland) when positive control sera reached a predetermined colour intensity. Antibody levels are expressed as absorbance values.

Statistical analyses - ELISA results were analysed by analysis of variance (ANOVA) and Student's t-test. Skin test results were analysed using a split unit design in a general linear model procedure on a statistics package (SAS Institute Inc., Cary NC, USA).

RESULTS

Clinical examination - All dogs examined showed signs of flea infestation manifested either as the presence of fleas or flea excreta or both. Seven dogs exhibited skin lesions typical of FAD, had fleas and flea excreta present and were thus clinically diagnosed as having FAD.

Reactions to feeding fleas - The 7 dogs clinically diagnosed positive for FAD (group 2) all reacted to feeding fleas by developing multiple small wheals at the site where fleas were fed. Of 11 dogs with no signs of FAD (group 3), 9 did not react to feeding fleas while 2 developed positive reactions. The 6 pups (group 1) did not react to feeding fleas (table 1).

Titration of flea antigens by intradermal injection - Intradermal injection of dogs with various concentrations of flea antigens (FS, FM) resulted in responses which were readily identifiable as wheals with diffuse erythema in some dogs. The average wheal diameter declined with decreasing concentrations of antigen injected (see figure 2 for responses to FS) at all times results were read. The responses to FM were less consistent than those to FS and showed a less obvious dose response effect (data not shown).

Reactions of groups of dogs to feeding fleas and to intradermal injection with antigen - The group of dogs which reacted to feeding fleas (flea-feeding positive dogs) also responded 15 minutes after injection of FS, FM and c-antigen at significantly greater levels than dogs which did not react to feeding fleas (flea-feeding negative dogs) ($p < 0.01$ for c-antigen, $p < 0.001$ for FS and $p < 0.005$ for FM); mean wheal diameter of c-antigen in flea-feeding positive and negative dogs were 3.9 ± 0.83 mm and 0.58 ± 0.14 mm respectively. There was no difference in the responses to either FS, FM or c-antigen by the flea-feeding negative dogs and the pups (mean wheal diameter of c-antigen in pups was 2.12 ± 1.6 mm). No differences were apparent between any of the groups 30 minutes and 1 hour after injection of antigens.

Of the dogs clinically diagnosed with FAD (n=7), all

were flea-feeding positive and 2 reacted to c-antigen, none to FM while 4 reacted to FS (table 1).

Flea-feeding negative dogs - Dogs in this group (n=9) did not react to any antigens injected 15 minutes after exposure. After 30 minutes, blebs of the buffer control injections had been resorbed in some dogs resulting in skin test which were variably positive. At 1 hour, all negative control blebs had disappeared while the antigen wheals were resorbed inconsistently.

Flea-feeding positive dogs - Six of nine dogs which reacted to flea-feeding also reacted to FS in concentrations of 1 µg/dose at 15 minutes; three of these dogs were also positive to c-antigen and one to FM at a concentration of 0.6 µg/dose. Observations similar to those made in dogs negative to feeding fleas were made at 30 minutes and 1 hour regarding buffer control and antigen wheals.

Pups - One pup reacted to c-antigen, FS at all concentrations and to FM at concentrations ≥ 0.6 µg/dose 15 minutes after injection (table 1). After 30 minutes, observations regarding buffer controls and antigen wheals were the same as those made in dogs. After 1 hour, the pup which was positive at 15 minutes was still positive to c-antigen and FM and FS at concentrations ≥ 1 µg/dose whilst no wheals were apparent in the other pups.

No reactions were apparent in any dog/pup 24 hours after either injection of antigen or feeding of fleas.

ELISA - IgG antibody levels against FS differed significantly between the flea-feeding negative dogs and the pups only ($p < 0.05$) (figure 3). There were no differences between the three groups of dogs in the IgG antibody levels against the antigens WF, c-antigen and FM. No differences could be established in IgE antibody levels against any of the antigens used (see figure 4).

DISCUSSION

Skin testing by injecting flea antigens intradermally is used as a diagnostic test for FAD, although more frequently clinicians diagnose FAD on the clinical presentation of the patient and on the response

to flea control. The reliability of skin tests for diagnosis of FAD was examined by different authors and contradicting results were obtained (Baker 1971; Van Winkle 1981). Baker (1971) used a crude flea extract suspended in phenol saline as antigen and found positive skin reactions in 73% of dogs in which FAD had been diagnosed. Van Winkle (1981) evaluated four commercial flea antigens; two of which were glycerinated, gave invalid results due to the skin being irritated by the glycerin component and the two aqueous extracts of antigen did not show significant test results in dogs with or without clinical FAD. However, in both studies the results of the skin tests with flea antigens were compared with the diagnoses made following clinical evaluation only and the reliability of these clinical diagnoses was not considered. We therefore used a provocation test as a positive control; fleas were fed on dogs and the reactivity to this test was then mooted to reflect the true flea allergic status of the dogs. All dogs with skin lesions, putatively diagnosed as FAD, reacted to feeding fleas while none of the pups exhibited reactions. However, two dogs with no clinical evidence of skin lesions or dermatitis reacted to feeding fleas. These dogs may either have been in the early stages of developing a hypersensitivity to fleas or may have had an IgE antibody response to flea antigens without having developed the full pathology which manifests itself as allergic dermatitis. This is supported by the observation (in Muller 1989) that clinical FAD rarely develops in pups under 6 months of age. We propose these two dogs were likely to develop clinical FAD on more prolonged or heavy exposure to fleas.

It is usually recommended (for example see Halliwell 1986) that intradermal skin tests be evaluated 15 to 20 minutes after injection or antigen. This was confirmed as the optimum time to read tests in our experiments: the blebs caused by injecting buffer resorbed inconsistently from 30 minutes to 1 hour and caused variability problems in evaluating the skin tests at these times.

The antigens used in this study did not correlate directly with allergic responses to feeding fleas which we propose as being the most correct assessment of either the presence of FAD or a predilection or predisposition to the disease. Of the three antigens tested (FM, FS, c-antigen), FS related best to both the responses of dogs to feeding fleas and the clinical diagnosis of FAD. Six dogs (67%) were positive to FS (15 minutes) out of the 9 which reacted to feeding fleas (see table 1) whereas only 3 dogs (33%) in this group were positive to the commercial antigen and 1 (11%) to FM. Dogs with clinical FAD were diagnosed positive by skin test at levels of 57%, 29% and 0%, using FS, c-antigen and FM respectively.

A typical dose response effect was not obtained with FM in the range of 0.03 µg to 1.25 µg, in contrast with FS. FS, a soluble antigen, would have better properties for diffusing from the site of injection than FM which is particulate.

The levels of canine IgG and IgE antibodies to flea antigens in dogs with clinically diagnosed FAD were measured by Halliwell and Longino (1985) using RIA; higher mean levels of antibodies of both isotypes were found in dogs with dermatitis than in a group of unaffected dogs. We found no relationship between IgG and IgE antibody levels to WF, FM, FS and either clinical evidence of FAD or reactivity of dogs to feeding fleas. The marker antibody used in the ELISA for IgG was specific for both heavy and light chains of IgG and thus may have obscured differences in the levels of this particular isotype between the groups of dogs. Some cross-reactivity with other isotypes was also reported for the marker antibody for IgE (Kirkegaard and Perry, pers. comm.). A blocking antibody, specific for heavy and light chains of IgG, was included in the ELISA in an attempt to minimize the cross-reactivity but it is recognized that this blocking antibody may have affected measurable IgE antibody levels through steric hindrance.

Pups (<6 months) had lower levels of antibodies to flea antigens then mature dogs but the differences were

only significant for IgG antibodies to FS compared to dogs with no response to feeding fleas. All of these dogs and pups had been exposed to fleas since fleas or flea excreta were found on all animals. Brisbane has a subtropical climate where fleas are a major problem for pets and the exposure of the dogs in this study to fleas might also be expected to be high as they were neglected dogs, obtained from a pound.

Table 1: Results of skin tests and provocative testing in dogs and pups with and without FAD diagnosed by clinical examination

	DOGS			PUPS
	FAD	NON-FAD	NON-FAD	NON-FAD
	REACTION TO FLEA-FEEDING			
ANTIGEN	POSITIVE (n=7)	POSITIVE (n=2)	NEGATIVE (n=9)	NEGATIVE (n=6)
RESULTS AT 15 MIN				
c-antigen	2*	1	-	1
FM (0.6 µg)	-	1	-	1
FS (1 µg)	4	2	-	1
RESULTS AT 30 MIN				
c-antigen	4	1	2	5
FM (0.6 µg)	2	2	2	3
FS (1 µg)	5	2	5	3
RESULTS AT 1 HOUR				
c-antigen	4	1	3	1
FM (0.6 µg)	-	1	1	-
FS (1 µg)	5	1	3	1

* Figures denote number of dogs with positive reactions. A skin test was considered positive when the average diameter of the wheal resulting from injection of antigen exceeded by 5 mm the average diameter of the wheal resulting from injection of the buffer control.

Example 2: Demonstration of the repeatibility of IDST with FS antigen and control preparations.

PBS, histamine phosphate 1:100,000 w/v; FS antigen, sterile water and Greer allergen were injected in triplicate in each of 4 dogs. These dogs had been prepared, skin tested and scored for their response to feeding fleas and clinical signs of FAD as described in example 1 and previously. The diameters of wheals at the site of injection were measured after 15 and 30 minutes. An average value for each response (n=3) was calculated. The variation in triplicate readings was classified as differences: ≤ 1 mm; ≥ 1 mm; ≥ 2 mm; ≥ 3 mm.

Most of the responses (83.3%) to injections varied less than 2 mm from their corresponding injections. See table 2 below.

Table 2: Variations in wheal diameters of 48 triplicate injections in IDST

Variations	Number of Injections	Percentage
< 1 mm	30	62.5
≥ 1 mm < 2 mm	10	20.8
≥ 2 mm < 3 mm	6	12.5
≥ 3 mm	2	4.2

Example 3: FS used in IDST in dogs in a definitive experiment.

MATERIALS AND METHODS

FS-Antigen (500 µl 3600 µg/ ml) maintained at 4°C was applied in each injection onto the HPLC column (described previously). The separated antigens were divided into six fractions designated (A,B,C,DE,F,G, see 4.4). Equal volumes of the fractions A, B, C, DE and F were combined to form fraction T. The protein concentration of all fractions was determined by a modified Lowry assay. The protein contents in fractions F and G could not be measured because the protein content was below the sensitivity of the Lowry assay. Separated fractions were

stored at -70°C.

In accordance with preliminary work with FS-Antigen a dose of 60 µl at 1 µg/ 60 µl was used for FS-Antigen and the T-Fraction in IDST. Fractions A,B,C, and DE were administered in doses which reflected their percentage representation in the FS-Antigen chromatogram. These antigen peaks were cut out and weighed. Consequently the concentrations of protein used for IDST were determined to be:

A	28.08%	: 0.28 µg/ 60 µl
B	24.08%	: 0.24 µg/ 60 µl
C	32.64%	: 0.33 µg/ 60 µl
B	15.2%	: 0.15 µg/ 60 µl

Fractions F and G could not be subjected to these calculations due to their low protein concentration and were used in IDST as collected from the HPLC separation. Fractions were diluted in freshly prepared PBS within 2 hours of carrying out IDST. Diluted antigens were stored at 4°C until injected (< 2 hours).

IDST were carried out as described previously. Each injection was done with a new needle. Injections were judged to be intradermal where a distinct bleb occurred: inaccurate injections were repeated immediately, until the expected bleb was visible. Histamine phosphate diluted with $_dH_2O$ was used as a positive control. The negative control was PBS. Reactions to the injected agents were measured with a calliper ruler at 15 and at 30 minutes. Most wheals did not show a clearly round circumference. Therefore all reactions were measured in 2 directions (minimal and maximal bleb diameters) and an average value of those measurements was calculated. A flea feeding test was performed at the same time as the IDST. Fleas were killed after the test by exposure to -70°C and were examined under a dissecting microscope (Olympus Optical Co. Ltd., Tokyo, Japan) for evidence of a blood meal, by dissecting each individual flea on white paper [illegible] spot on the paper indicated the

particular flea had fed. The fleas were also examined for the presence of *D. caninum* cysticercoids. DTH reactions were assessed by evaluating injection sites 24 hours after the IDST procedure.

Thirtythree dogs, 16 flea feeding negative (ff-) and 17 flea feeding positive (ff+), were tested as described above using FS-Antigen and HPLC-fractionated antigens. In addition, Greer-Flea antigen at a dilution of 1:1,000 w/v was applied. Histamine phosphate at a concentration of 1:10,000 w/v was injected as a positive control in 24 dogs. All dogs (33) received histamine phosphate at a concentration of 1:100,000 w/v. PBS was used as a negative control.

Skin test readings obtained from all dogs was assessed, using an objective grading system frequently used in the literature. The mean diameter of the antigen wheal (measured at 15 and 30 minutes) was compared with the mean diameter of the bleb at the site of the negative control. Reactions that were ≥ 5 mm larger than the negative control bleb were considered to be positive.

Antibody levels in dogs were measured using an indirect enzyme-linked immunosorbent assay (ELISA).

RESULTS

Blebs in a range of 5 to 7 mm in diameter were apparent upon injection of the negative control solution (PBS) in all dogs. None of the dogs exhibited a wheal and flare reaction to the PBS. Complete resorbtion of the PBS occurred at various times in different animals, usually 30 to 60 minutes after the IDST procedure.

Injection of histamine at 1:100,000 caused an average skin reaction of 10.6 ± 0.2 mm (x ± SEM) (range: 8.5 to 13.8 mm) at 15 minutes, and 10.8 ± 0.2 mm (x ± SEM) (range 8.5 to 14.3 mm) at 30 minutes. Histamine diluted to a concentration of 1:10,000 caused average reactions of 13.6 ± 0.3 mm (x ± SEM) (range: 10.5 to 15.5 mm) at 15 minutes and 14.4 ± 0.4 mm (x ± SEM) (range: 11.3 to 17.3 mm) at 30 minutes after injection.

Immediate reactions to flea antigens consisted of erythematous (in animals with either non- or poorly

pigmented skin) wheals, without pseudopodial reactions in all cases. The results for immediate IDST reactions are shown in Table 3 below. Dogs were grouped according to their reactions to feeding fleas (ff+/ ff-) and subgrouped in accordance with the presence of clinical signs (FAD+/ FAD-). The results are given as the percentage of positive responses in either a group or clinical subgroup. Only one ff- dog had FAD, therefore the percentage response for this subgroup was not calculated.

Only two dogs (both ff+) exhibited clear DTH reactions to FS-Antigen, Greer-Flea Antigen and Fraction-T. In one of the dogs DTH reactions also appeared at the injection sites of Fractions-B, C and DE. The DTH reactions consisted of red indurated nodules of 2 to 3 mm diameter.

Multiple blebs were visible at the flea feeding site of both dogs after 24 hours.

Antibody levels to the antigens tested did not differ significantly between the 2 groups of dogs (see Table 4).

Table 3: Interpretation of the IDST. A result was considered positive when the mean wheal diameter of the antigen injection site was ≥ 5 mm than the mean wheal diameter of the negative control site. Results are expressed as the percentage of dogs with positive responses in the group. HPLC fractionated antigens (A-T); FS-Antigen (FS-AG); Greer-Flea antigen (Greer); not calculated (nc).

	Response of dogs to feeding fleas					
	positive			negative		
	clinical signs			clinical signs		
Antigen	total n=17	FAD + n=9	FAD - n=8	total n=16	FAD + n=1	FAD - n=15
Greer	41.2	22.2	62.5	12.5	nc	13.3
A	-	-	-	-	nc	-
B	35.3	22.2	50	-	nc	-
C	58.8	66.7	50	-	nc	-
DE	-	-	-	-	nc	-
F	35.3	44.4	25	-	nc	-
G	11.8	11.1	12.5	-	nc	-
T	76.5	66.7	87.5	6.3	nc	6.7
FS-AG	94.1	88.9	100	-	nc	-

Measurement of anti-flea antibody levels of dogs by ELISA

No significant differences in the mean anti-flea antibody levels occurred between ff+ and ff- dogs for any of the antigens tested (see Table below). A trend was apparent in that the ff+ group had slightly higher antibody levels against all flea antigens tested.

Table 4: Mean levels of antibodies of ff+ and ff- dogs against HPLC fractionated antigens (A-T) and FS-Antigen (FS-AG). Levels were measured by ELISA and are expressed as mean optical density units ± SEM (x ± SEM), measured at 405 nm.

Antigen	Flea feeding positive (x ± SEM)	Flea feeding negative (x ± SEM)
A	0.271 ± 0.034	0.250 ± 0.018
B	0.096 ± 0.013	0.073 ± 0.009
C	0.034 ± 0.006	0.025 ± 0.006
DE	0.033 ± 0.008	0.022 ± 0.005
F	0.009 ± 0.004	0.006 ± 0.003
G	0.007 ± 0.003	0.005 ± 0.002
T	0.116 ± 0.017	0.090 ± 0.009
FS-AG	0.141 ± 0.015	0.119 ± 0.010

In this study 94% of flea feeding positive dogs were detected when FS was used as an antigen in intradermal skin tests. No false positives were identified with this antigen. FS is highly specific and sensitive for detecting dogs with FAD. A grading system that defines a positive reaction as ≥ 5 mm than the negative control is appropriate for evaluating reactivity to flea antigens.

Clinical separation of FAD+ and FAD- animals must be viewed with suspicion. In particular because many FAD negative animals had positive reactions to feeding fleas. For this reason, the response to feeding fleas was used as the criterium of whether or not a dog was hypersensitive to fleas. It is important to note that this form of provocative testing has not been used frequently to establish the hypersensitivity status of dogs; more usually clinical evidence of FAD is used to categorize animals when testing diagnostic preparations. It is clear from the results reported here that clinical separation of "allergic" dogs is not useful for distinguishing between hypersensitive and normal dogs and

should not be used to judge grading systems or flea antigens.

None of the antigens separated on HPLC were satisfactory in detecting hypersensitive dogs. Neither did Greer antigen yield good results. The best results with Greer antigen were obtained using a method for interpreting results which was similar to that recommended by the manufacturer. A total of 41.2% of flea feeding positive dogs were recognised as positive with 12.5% false positive dogs when Greer antigen was used in IDST. These results are similar to those obtained by Reedy (1986).

Example 4: Diagnosis of flea allergy dermatitis in cats using FS and various other flea antigens in intradermal tests.

Few studies on the responses of cats to intradermal tests with antigens have been conducted to establish the presence of allergy (Kieffer and Kristensen 1979; Reedy 1982; Kunkle and Milcarsky 1985). The value of the intradermal test using flea antigens to diagnose flea allergy dermatitis (FAD) is inconclusive. We evaluated the responses of cats with and without clinical signs of FAD to feeding fleas and to intradermal injection of various flea antigens.

Six adult female and five adult male cats were tested; the cats were domestic shorthaired (10) and a persian crossbreed (1). The [illegible] examined for clinical signs of FAD (Muller et al. 1989) and grouped accordingly. Unfed fleas (15), *Ctenocephalides felis felis*, were fed on cats and the site of feeding was examined 15 minutes after exposure for reactions (palpable and visible wheals and erythema) as previously described. A commercial flea antigen preparation (Greer antigen (Greer Laboratories, Inc, Lenoir, NC, USA) was obtained.

Membrane antigens (FM) and soluble antigens (FS) were prepared from whole adult fleas as described previously. Salivary glands and guts were dissected from live adult fleas and flea salivary gland antigens (SG)

and flea gut antigens (GUT) were prepared by homogenizing the organs in phosphate buffered saline (PBS), pH 7.2 using a glass homogenizer, sonicating the homogenized organs for 20 seconds at an amplitude of 21 microns. The homogenized organs were centrifuged for 20 minutes at 15,000 g. The resulting pellet was rehomogenized in an aliquot of the supernatant, sonicated as described above after adding the rest of the supernatant. The resulting preparations were designated as GUT and SG, respectively. Cats were injected intradermally with 60 µl of different concentrations of the various flea antigens and with 1/100,000 w/v histamine phosphate and with PBS. The resulting wheal diameters were measured twice, horizontally and vertically, 15 minutes after injection. A test was considered positive when the average wheal diameter at the site of injection of an antigen exceeded the average wheal diameter of the negative control (PBS) by 5 mm.

All cats with clinically diagnosed FAD (n=5) reacted to feeding fleas and to intradermal injections with flea antigens; no reactions were observed in cats (n=6) with no signs of FAD to either flea-feeding or intradermal injections with flea antigens (table 5).

All cats which reacted positively to flea-feeding also reacted positively to Greer antigen, FS at 2µg/dose and SG at 1µg/dose (table 5). Lower concentrations of these antigens induced variable reactions. Injection with FM (2µg/dose) failed to detect one of the cats which had reacted positively to flea-feeding. Two cats had positive reactions to gut antigens.

All the antigens used in the test were highly specific; this finding is in concordance with the findings of Kieffer and Kristensen (1979) who also reported high specificity when a crude whole flea extract was used in intradermal tests of cats for FAD. Positive reactions to intradermal tests with Greer antigen have been also described in previous work where cats with miliary dermatitis reacted all to the antigen (Kunkle and Milcarsky 1985). The results reported for cats here and

in the literature, contrast with those obtained in dogs; in similar studies of 9 dogs responsive to feeding fleas, 67% were identified as reactive to FS, 11% to FM and 33% to Greer antigen.

Gut was tested as an antigen as it is a possible source of allergens to which the host is naturally exposed through regurgitation of gut contents. Although two cats reacted to the gut antigen, the results of the skin tests did not support the presence of significant allergens in the gut at the doses tested.

Oral secretions derived from *Ct. felis felis* have been shown to induce hypersensitivity in guinea pigs (Benjamini et al. 1963). In the study reported here, cats were injected with extracted salivary glands in an intradermal test and no healthy cat reacted to the injection of these antigens. Only four of the cats which had FAD and which reacted to feeding fleas were tested with SG; all of these cats reacted, confirming the presence of allergens in flea salivary glands.

All cats with FAD reacted to injection of FS and Greer antigen. FS a soluble antigen derived from whole fleas and Greer antigen an aqueous extract of fleas contain salivary gland antigens. As FS is readily obtainable compared to SG, FS is the preferred antigen for intradermal tests.

Flea antigens can be used in intradermal tests to diagnose FAD in cats; in the limited study reported here, specific and sensitive assays were demonstrated using FS, Greer antigen and SG as antigens. The efficacy of this test in cats is in contrast with its value in dogs where false positive and negative reactions negate the value of the test in diagnosis of FAD.

Table 5: Number of cats reacting positively to intradermal testing with different flea antigens.

ANTIGENS (µg/DOSE)		REACTIONS OF CATS TO FLEA FEEDING	
		POSITIVE (n=5)	NEGATIVE (n=6)
GREER (1/1000 w/v)		4*	-
SG	1	4+	-
	0.5	4	-
	0.2	2	-
FM	2	4	-
	1	1	-
	0.2	1	-
FS	2	5	-
	0.5	3	-
	0.2	4	-
GUT	2	1	-
	0.2	2	-

* Figures denote number of cats with positive reactions. A skin test was considered positive when the average diameter of the wheal resulting from injection of antigen exceeded by 5 mm the average diameter of the wheal resulting from injection of the buffer control.

\+ Only 4 cats were tested with SG at 1 µg/dose.

Example 5: Electrophoresis of FS

The proteins of the FS antigen preparation (FS-Ag) were separated on a denaturing polyacrylamide gel using standard techniques. The separating gel consisted of a 7.5% stock acrylamide mixture (30 parts acrylamide:8 parts bis-acrylamide) diluted in 1.5 M Tris-Cl buffer pH 8.8 + 1% SDS, and polymerised with TEMED and APS. Minigels (1.5 x 70 x 80mm) were poured. A stacking gel (5mm high) consisting of 4% acrylamide mixture in 0.5 M Tris-Cl pH 6.8 and 1% SDS, polyermised with TEMED and

APS, was poured on top of the polymerised separating gel.

For electrophoresis, the FS-Ag was diluted to 0.5 mg/mL with sample buffer (1 part FS-Ag:4 parts sample buffer), incubated at 95°C for 5 min, and appropriate, typically 25µL, aliquots applied to the wells in the gel. High and low molecular weight markers were run with the FS-Ag on the gel. The gel was double-stained with Coomassie blue followed by silver staining.

RESULTS

The stained gel is presented in Figure 5. FS-Ag was found to contain two very high MW proteins, one having a MW slightly greater than 200 000, the other somewhat higher. Also evident were a few faint bands with MW between 45 000 and ~ 70 000; more prominent bands at 46 000, 40 000 and 31 000, and other faint bands from 66 000 down to the base of the gel.

Example 6: Antibody Detection using Immunoblotting

The FS antigen preparation was subjected to polyacrylamide gel electrophoresis as described in Example 5 with the exception that the material for electrophoresis, typically 300µL, was loaded in a single long trough across the gel. The FS antigen proteins separated on polyacrylamide gels were transferred to nitrocellulose paper (NCP) immediately after electrophoresis. Transfer was done at 4°C using 0.7-1 A current for four hours in an LKB Transphor apparatus. After transfer, the NCP was blocked with 5% low fat milk powder in PBS-0.05% Tween (PBS-T) pH 7.2 at 37°C for one hour, after blocking with PBS-T the NCP was transferred into a Miniblotter (Immunetics) which had multiple incubation lanes.

For dog IgG antibodies, serum from animals was applied in doubling dilutions to separate lanes of the blotter (85 µL per lane, serum diluted in PBS-T). The apparatus was then incubated at 37°C on a rocking platform for 1 hour. Serum was aspirated from the lanes, the NCP removed from the blotter and washed 4 x 8 min in PBS-T. The NCP was then incubated for 1 hour at 37°C with biotinylated rabbit anti-dog IgG (H and L chain)

Jackson Research Laboratories (JIRL) diluted 1:1000 in PBS-T/1% gelatine (PBSTG). After a further 4 x 8 min washes in PBS-T the NCP was incubated for one hour at 37°C with peroxidase-conjugated streptavidin (JIRL), diluted 1:1000 in PBSTG, followed by 2 x 4 min washes in PBS-T and 2 x 4 min washes in PBS after which substrate (4-chloro-1-naphthol in PBS + H_2O_2) was added. Appearance of bands was monitored and the reaction was stopped with distilled water. The blots were photographed within 48 hours.

For dog IgE antibodies, the NCP with bound transferred protein was placed in the blotting apparatus and sera diluted in PBS-T (1.30) were added to individual lanes. The apparatus was incubated for 2 hours at 37°C on a rocking platform. After aspirating the sera, the NCP was transferred to a small plastic incubation tray and washed with PBS-T 4 x 8 min. Twenty five mL of rabbit anti-dog IgE was added (1:500 in PBSTG), incubated 1 hour at 37°C and washed 4 x 8 min in PBST. Biotinylated goat anti-serum (JIRL) (diluted 1:500 in PBSTG) was added, incubated one hour at 37°C and washed 4 x 8 min prior to adding the final component, streptavidin HRPO (JIRL) diluted 1:1000 in PBSTG. After 1 hour at 37°C this was washed twice with PBST then twice with PBS, before adding the 4-chloro-1-naphthol + hydrogen peroxide substrate solution. Development of colour was monitored, and reaction terminated by washing the blot in distilled water. Photographs of the blot were taken within 48 hours.

Results

The results of the IgG blotting are presented in Figure 6. The first six lanes represent results from FF+ dog sera, and the next six lanes results from FF- dog sera. These sera have been arranged in decreasing order of [IgG] vs FS-Ag, as determined by ELISA. Thus serum from the FF+ dog with the highest [IgG] appears in lane 1 and the highest FF- dog serum in lane 7. As the photograph reveals, total levels of IgG vs FS-Ag do not necessarily correlate with strongly reactive sets of

bands in an immunoblot.

Figure 7 shows the results of screening sera from FF+ and FF- dogs for IgE antibodies against FS-Ag proteins. As with the IgG blots, common bands occur at >300 000, ca. 97 000, 66 000 and 46 000, but there are in addition common bands between 30-45 000 apparent in both FF+ and FF- lanes which were not apparent in the IgG blots.

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CLAIMS:

1. An antigen preparation from fleas of the genus *Ctenocephalides* characterised having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5.

2. An antigen preparation according to claim 1 further characterised as having an IgG immunoblotting pattern as shown in Figure 6.

3. An antigen preparation according to claim 1 further characterised as having an IgE immunoblotting pattern as shown in Figure 7.

4. An antigen preparation accordingly to claim 1 wherein said fleas of the genus *Ctenocephalides* are *Ctenocephalides felis* and/or *Ctenocephalides canis*.

5. An antigen preparation prepared by a process comprising the following steps:

a) preparing a total extract of fleas of the genus *Ctenocephalides*;

b) removing debris and cells from said total extract to provide a whole flea extract; and

c) separating a soluble flea antigen preparation from said whole flea extract.

6. An antigen preparation accordingly to claim 5 wherein said fleas of the genus *Ctenocephalides* are *Ctenocephalides felis* and/or *Ctenocephalides canis*.

7. A method of preparing an antigen preparation comprising the steps of:

a) preparing a total extract of fleas of the genus *Ctenocephalides*;

b) removing debris and cells from said total extract to provide a whole flea extract;

c) separating a soluble flea antigen preparation from said whole flea extract.

8. A method according to claim 7 wherein said fleas of the genus *Ctenocephalides* are *Ctenocephalides felis* and/or *Ctenocephalides canis*.

9. A method according to claim 7 wherein step (a) comprises the steps of homogenising said fleas and

sonicating the resulting homogenate.

10. A method according to claim 7 wherein step (b) comprises centrifugation of said total extract at approximately 600g.

11. A method according to claim 7 wherein step (c) comprises centrifugation of said whole flea extract at approximately 100,000g.

12. A method of detecting flea allergy in an animal animal, said method comprising the steps of:

a) intradermally injecting into an animal an antigen preparation from fleas of the genus *Ctenocephalides* characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5;

b) measuring the diameter of the wheal formed at the site of injection; and

c) comparing said wheal diameter with the diameter of wheals formed at the site of injection of a control solution to determine whether said animal is allergic to said fleas.

13. A method according to claim 12 wherein said flea allergy is due to infestation with fleas of the genus *Ctenocephalides*.

14. A method according to claim 13 wherein said fleas of the genus *Ctenocephalides* are selected from the group consisting of *Ctenocephalides felis* and *Ctenocephalides canis*.

15. A method according to claim 12 wherein said animal is selected from the group consisting of dogs and cats.

16. A method of hyposensitising an animal to flea allergy comprising administering to said animal an antigen preparation from fleas of the genus *Ctenocephalides*, said antigen characterised as having a high performance size exclusion chromatography profile as shown in Figure 1 and a denaturing polyacrylamide gel electrophoresis profile as shown in Figure 5.

17. A method according to claim 16 wherein said flea allergy is due to infestation with fleas of the genus

Ctenocephalides.

18. A method according to claim 17 wherein said fleas of the genus *Ctenocephalides* are selected from the group [illegible] *canis*.

19. A method according to claim 16 wherein hyposensitisation is for the treatment of flea allergy dermatitis.

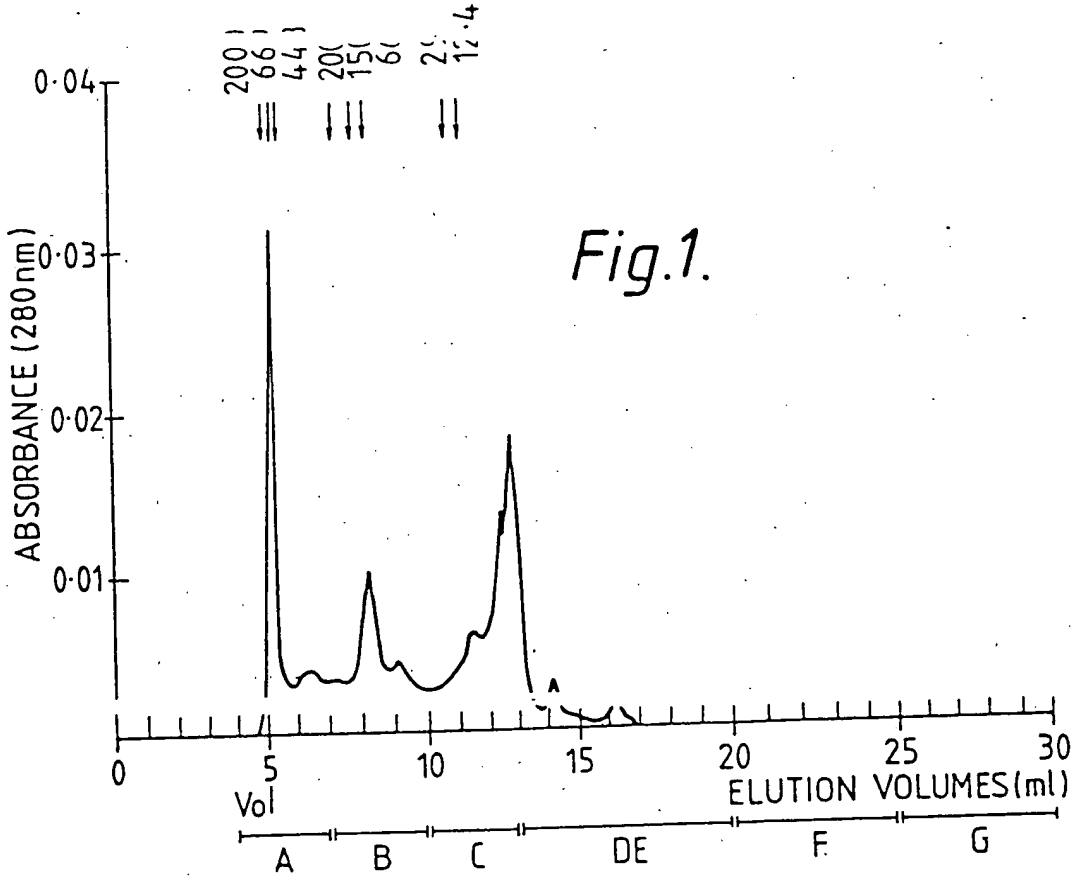


Fig.1.

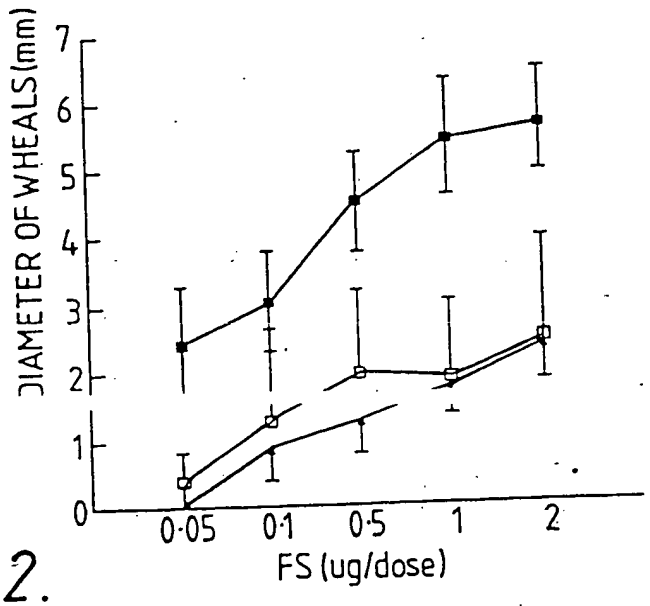
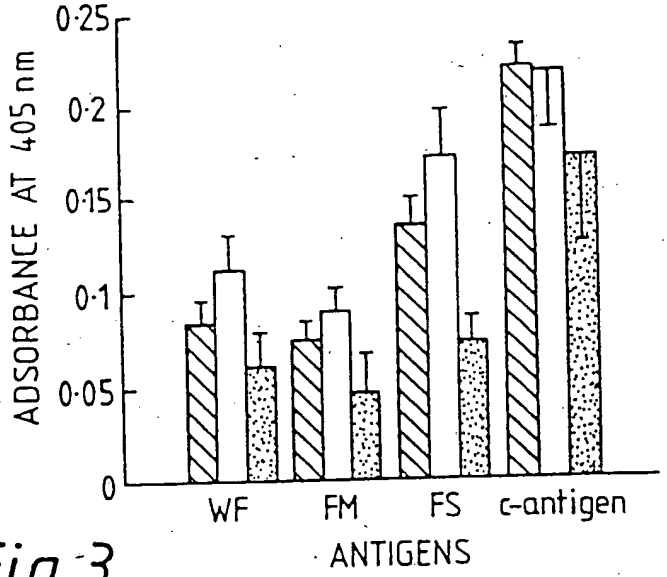


Fig.2.

SUBSTITUTE SHEET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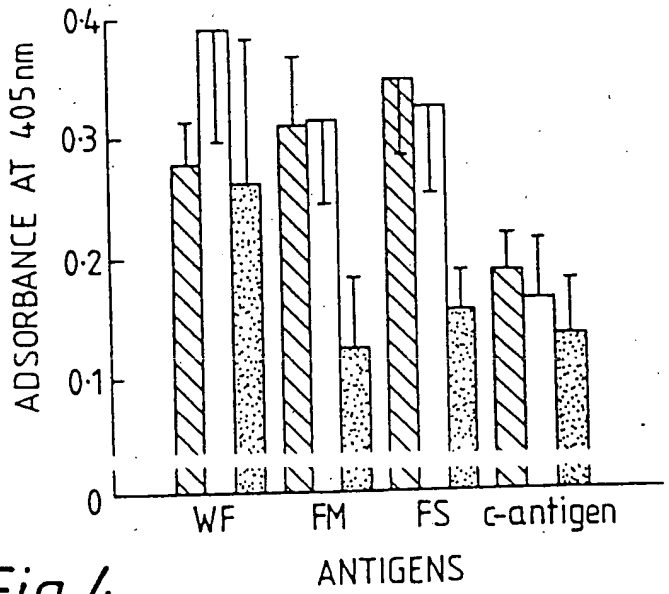
c-antigen
ANTIGENS

Fig. 3.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ANTIGENS

Fig. 4.

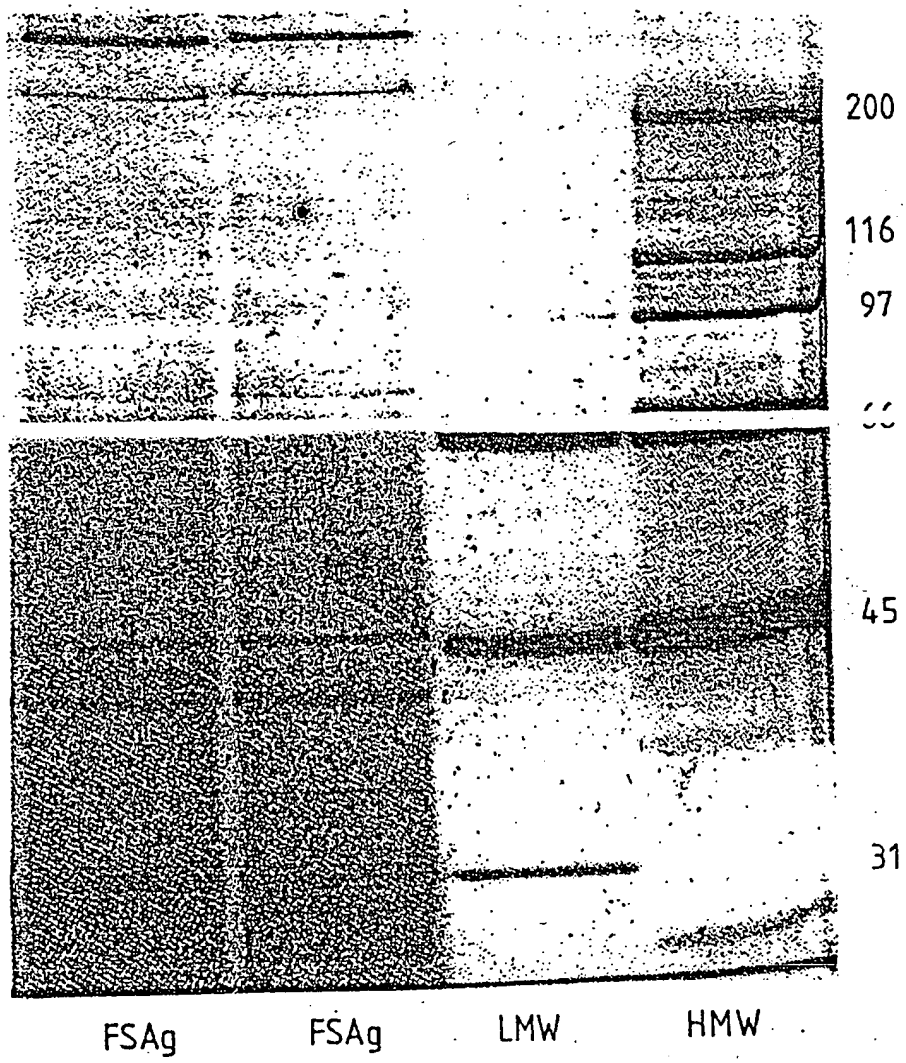

Fig. 5.

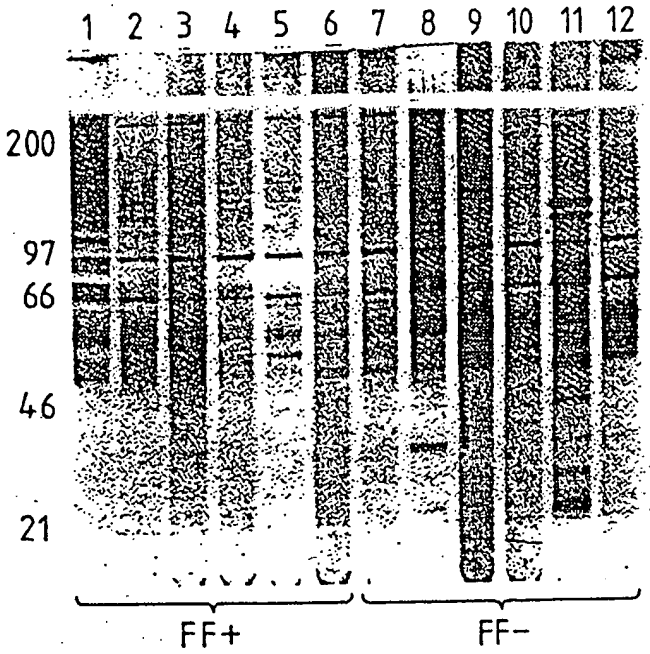

Fig.6.

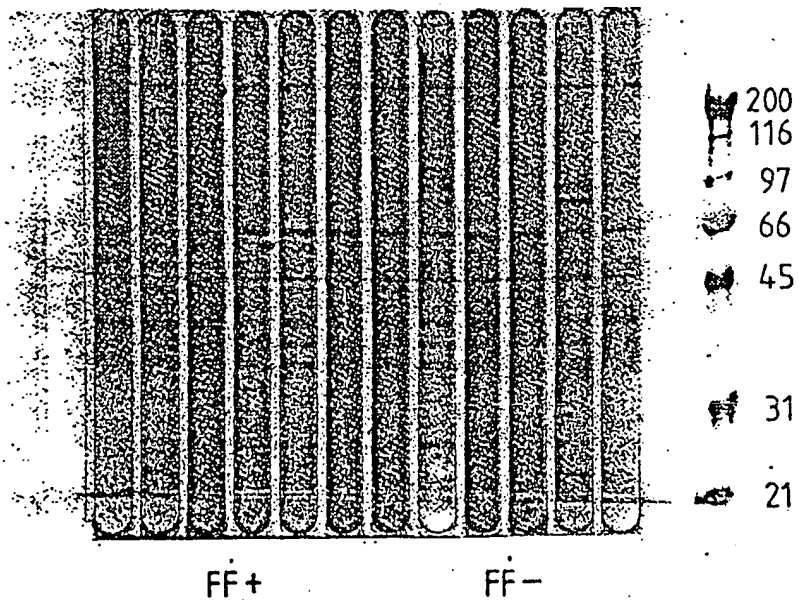

Fig.7.

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] A61K 39/00, 39/35, 35/64, C07K 15/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : A61K 39/00, 39/35, 35/64, C07K 15/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU : IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

DERWENT :

JAPIO : Flea # or Ctenocephal

CHEM ABS :

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	AU-A-85370/91 (PARAVAX, INC. AND THE UNIVERSITY OF QUEENSLAND) 5 March 1992 (05.03.92). See whole document.	5, 7, 9
A	AU-B-45936/85 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 13 February 1986 (13.02.86).	5, 7, 9
A	Chemical Abstract Vol. 109 No. 11 issued 12 September 1988 Columbus, Ohio, USA Yanni, I.M. et al. "Effect of AHR-5333 on flea antigen extract-induced skin reactions in flea-allergic dogs". Abstract No. 85921t.	5, 7, 9

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1993 (24.06.93)	29 JUNE 1993 (29.06.93)

Name and mailing address of the ISA/

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This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
AU	85370/91	WO	9203156
AU	45936/85	None	

END OF ANNEX